



Europäisches  
Patentamt

European  
Patent Office

Office européen  
des brevets

**BEST AVAILABLE COPY**

REC'D 07 JUL 2004

WIPO

PCT

Bescheinigung

Certificate

Attestation

Die angehefteten Unterla-  
gen stimmen mit der  
ursprünglich eingereichten  
Fassung der auf dem näch-  
sten Blatt bezeichneten  
europäischen Patentanmel-  
dung überein.

The attached documents  
are exact copies of the  
European patent application  
described on the following  
page, as originally filed.

Les documents fixés à  
cette attestation sont  
conformes à la version  
initialement déposée de  
la demande de brevet  
européen spécifiée à la  
page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

03076388.2

**PRIORITY  
DOCUMENT**  
SUBMITTED OR TRANSMITTED IN  
COMPLIANCE WITH RULE 17.1 (a) OR (b)

Der Präsident des Europäischen Patentamts;  
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets  
p.o.

R C van Dijk



Anmeldung Nr:  
Application no.: 03076388.2  
Demande no:

Anmeldetag:  
Date of filing: 09.05.03  
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

LABORATORI DERIVATI ORGANICI S.P.A.  
Via M. Barozzi, 4  
20122 Milano  
ITALIE

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:  
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.  
If no title is shown please refer to the description.  
Si aucun titre n'est indiqué se referer à la description.)

Process for the physical depolymerization of glycosaminoglycans and products  
obtained therefrom

In Anspruch genommene Priorität(en) / Priority(ies) claimed /Priorité(s)  
revendiquée(s)  
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/  
Classification internationale des brevets:

C08B/

Am Anmeldetag benannte Vertragstaaten/Contracting states designated at date of  
filing/Etats contractants désignées lors du dépôt:

AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LU MC NL  
PT RO SE SI SK TR LI

**Process for the physical depolymerization of glycosaminoglycans and products obtained therefrom.**

**State of the art**

5 Glycosaminoglycans are natural products of large pharmaceutical interest. Among the most widely used we can mention heparin, dermatan, heparansulphate and chondroitines.

The molecular weight of the natural products varies considerably and normally ranges from 5 to 40 kDa. It is however known that, for certain applications, lower molecular  
10 weights lead to higher pharmacological activity. The high molecular weight of these compounds often renders impossible their oral administration. Furthermore, it is known that specific activities of glycosaminoglycans are related to relatively short saccharide sequences. Thus, it would be very advantageous to depolymerize glycosaminoglycans reducing the molecular weight without losing the active sites present in the molecule.

15 The chemical depolymerization of glycosaminoglycans is well known in the art and leads to glycosaminoglycans of lower MW but also with a lower S content.

EP 394 971 discloses an enzymatic or chemical depolymerization process. The obtained heparin oligomers have a sulphur content lower than 9%.

WO 90/04607 discloses a depolymerization of heparin and dermatansulfate by the use  
20 of  $\text{H}_2\text{O}_2$  and  $\text{Cu}^{2+}$ . The ratio  $\text{SO}_3^-/\text{COO}^-$  is slightly lower than in the starting heparin.

US 4,987,222 discloses a method for the depolymerization of heparin by the use of gamma rays. The examples disclose the preparation of heparin of Mw around 5,000 Da and with a high S content. However, the heparin produced by this method presents a certain amount of degradation products as a result of uncontrolled side reactions.

25 It is therefore desirable to reduce the molecular weight of glycosaminoglycans without substantially modifying the chemical structure of the same.

It is known in the art that the irradiation of glycosaminoglycans with UV leads to their degradation. For example, in Polymer Photochemistry 6 (1985) 465-474, Khan et al. studied the effect of UV radiation on aqueous solution of heparin-Ca salts.

30 Depolymerization was detected, but it was only considered as a degradation process, not as a potential industrial process for the controlled depolymerization of heparin.

### Summary of the invention

The present invention relates to a physical process for the depolymerization of glycosaminoglycans by the use of UVC radiation having a peak in the range from 245 nm to 260 nm. It also relates to the glycosaminoglycans obtained by this process.

### Detailed description of the invention

The present invention provides a physical depolymerization process which reduces the molecular weight of glycosaminoglycans without substantially modifying the chemical structure of the same.

10 The objective is achieved through use of UVC radiation. When using heparin as a starting material, this process results in a low to ultra-low molecular weight heparin characterized by high S content.

The starting materials to be used in the process according to the present invention are natural glycosaminoglycans such as heparin, heparansulphate, dermatane and  
15 chondroitine. Other suitable starting materials are derivatives of glycosaminoglycans obtained by known methods. Thus, for example, the N-acetyl or N-sulphate groups of the residues of hexosamine can be transformed in amino groups through N-desulphation or N-deacetylation reactions and the sulphate groups of the uronic acids through desulphation reactions can give rise to epoxy groups.

20 In another embodiment, it is possible to use as a starting material for the process of the present invention a glycosaminoglycan which has already undergone a depolymerization process either chemical, physical or enzymatic. Examples of physical depolymerization methods that can be used in combination with the process of the invention are described in EP02425143.3 (LDO) and IT2002MI001372 (LDO), which  
25 are herewith incorporated by reference. The use of partly depolymerized glycosaminoglycans is for example relevant in case of heparin which has undergone an acid pretreatment that has as a side effect partial depolymerization, or when depolymerizing functionalized glycosaminoglycans. The conditions used for the introduction of functional groups are sometimes also causing reduction of the molecular  
30 weight of the polysaccharide.

Thus, not only it is possible to perform both steps by using UVC radiation, but it is possible to perform a first depolymerization step by using UVC radiation followed by a

second step using chemical-enzymatic depolymerization, or to perform a first step of chemical-enzymatic depolymerization followed by UVC radiation depolymerization.

The process of the present invention allows reduction of the molecular weight of the glycosaminoglycane without sensible modification of the chemical structure of the polysaccharide. The Mw of the glycosaminoglycane after irradiation is equal to or lower than 50% of the Mw of the glycosaminoglycane prior to irradiation

Absorption by a molecule of radiation in the ultraviolet (200-400 nm) or visible (400-800 nm) region of the spectrum can result in an excited state so high in energy that the energy absorbed is comparable in magnitude with the bond dissociation energies associated with organic molecules. If absorption occurs at 250 nm, the energy associated with this transition ( $E=480 \text{ kJ mol}^{-1}$ ) is greater than the bond dissociation energies of a carbon-carbon  $\sigma$ -bond ( $D\approx 347 \text{ kJ mol}^{-1}$ ), a carbon-oxygen  $\sigma$ -bond ( $D\approx 330 \text{ kJ mol}^{-1}$ ) and a carbon-hydrogen bond ( $D\approx 414 \text{ kJ mol}^{-1}$ ), average values in polyatomic molecules. It is not surprising therefore that chemical reaction can be induced by excitation with ultraviolet light. Generally speaking, homolytic cleavages of a covalent bond in organic molecules can be induced by UV radiation, forming two intermediate radicals.

Mercury vapour arc lamps are now the source of choice for most photochemical reactions in solution phase. These sources provide radiation in the ultraviolet and visible parts of the spectrum covering a range from 200 nm to 750 nm and so are useful for practically all purposes. There are three main type of mercury lamps designed as low, medium and high pressure and each has a different characteristic. The low pressure lamp operates at room temperature and mainly emits at 253.27 and 184.9 nm. Medium pressure lamps exhibit a weak continuum with a superimposition of spectral lines, associated with a diminished intensity at 253.27 and 184.9 nm. For the high pressure lamp, the large increase in pressure introduces many more lines. The emission below 280 nm is very weak. 189.4 nm radiation (far UV) is absorbed by the solvent, water. When absorption occurs at 184.9 nm, the energy associated with this transition is  $E=649 \text{ kJ mol}^{-1}$ . As the bond dissociation energy of oxygen-hydrogen is  $\approx 455 \text{ kJ mol}^{-1}$ , average value in polyatomic molecules, this radiation can generate  $\text{H}^\cdot$  and  $\text{OH}^\cdot$  from

water. 194.2 nm (far UV) and 253.27 nm are absorbed by the heparin molecule. Above 300 nm, heparin does not absorb.

The present invention relates to the use of UV radiation having an emission peak in the range from 245 nm to 260 nm, preferably from mercury lamps, most preferably low and medium pressure lamps.

In another embodiment the present invention is directed to the use of UV radiation prevalently at the wavelength of about 253 nm. In fact, it is possible to use filters that absorb the radiation at different wavelength and let pass only the 253 nm radiation. In this way it is possible to minimize side reactions as described before and obtain a high selectivity in the depolymerization reaction.

Without limiting the scope of the invention, it is believed that the radiation at 253 nm can produce dissociation of glycosidic bond and this is believed to be an important step in the depolymerisation. The excited molecule releases energy by forming two intermediate radicals, that somehow evolve to stable structures of lower  $M_w$ .

Although the mechanism of depolymerization is still unclear, we suppose that the formed free-radicals can be trapped by the oxygen present in water, and the resulting oxygen-oxygen bond can be cleaved with a mechanism similar to common autooxidation induced by atmospheric oxygen and sunlight (oxygen-oxygen  $\sigma$ -bond:  $D \approx 137 \text{ kJ mol}^{-1}$ ). We can describe this pathway to depolymerization,  $\text{C-O-C} \rightarrow \text{C-OH} + \text{C-OH}$ , as autooxidation of glycosidic bonds.

However, the process can be performed also in the absence of oxygen, indicating the presence of other mechanisms of evolution of the free-radicals which do not require reaction with oxygen.

The process of the invention is usually performed on a aqueous solution of the glycosaminoglycane.

The concentration of glycosaminoglycane in the solution can vary in a broad range. Preferably it is comprised between 2 and 25% w/v, more preferably between 4 and 15%. The pH of the aqueous solution is preferably kept in the range of 3.0 to 7.0, most preferably between 4.0 and 6.0. In fact, when the solution is basic, anionic depolymerization of the glycosaminoglycane takes place.

To regulate the pH to the desired value, it is possible to use weak acids such as acetic acid, citric acid and the like.

After irradiation, the solutions is very clear and does not require any treatment to remove colored substances.

It is also possible to fractionate the intermediate depolymerized glycosaminoglycane by Gel Permeation Chromatography. The fractions containing the desired molecular weights are collected, concentrated by ultra filtration and lyophilized.

The process of the present invention is preferably performed by using a dynamic irradiation process.

With the term "dynamic irradiation process" it is meant a process wherein the solution to be irradiated is circulating as a thin layer in a lamp jacket and then returns to a reservoir where it is preferably thermostated. The liquid can circulate in one or more lamps which can be connected in series or in parallel.

The flow-rate of the solution in the circuit is not critical, but it is preferred to have a flow-rate high enough to avoid overheating of the solution.

The temperature of the solution in the circuit can vary in a broad range. Preferably it is maintained between 0 and 70 °C, more preferably between 10 and 50°C.

The process can be performed either in batch or in continuous mode. The apparatus is preferably formed of a reservoir from which the liquid moves to the irradiation area.

The liquid is then returned to the reservoir.

In another embodiment of the invention, the solution is continuously withdrawn from the reservoir and subjected to membrane filtration with a cut off that can vary according to the desired target of MW.

For example, when willing to obtain depolymerised heparin with a  $M_w$  comprised between 2,000 and 3,000, it is possible to use a cut off of 5,000 Da. If the desired  $M_w$  is below 2,000 Da, then it is possible to use a cut off of 3,000 Da.

By the use of this continuous membrane filtration step, the solution which undergoes irradiation is maintained at a higher  $M_w$ . It is therefore possible to avoid formation of very small heparin fragments which present lower pharmaceutical activity.

### Experimental section

Molecular mass ( $M_w$ ) was determined by size exclusion chromatography (European Pharmacopoeia 4<sup>th</sup> ed.: 2.2.30 and 2.2.46 for chromatography techniques and 01/2002:0828 p.1297 for method).

Absorbance at 260 nm was determined according to European Pharmacopoeia 4<sup>th</sup> ed 01/2002:0828 p.1297.

Anti Xa activity was determined according to the method described in European Pharmacopoeia 4<sup>th</sup> ed.: 2.2.30 and 2.2.46 for chromatography techniques and 01/2002:0828 p.1297 for method.

Anti coagulant activity was determined according to the method described in European Pharmacopoeia 4<sup>th</sup> ed.: 2.7.5 pg 168.

### Example 1

10 5 l of a 10% solution of heparin Na salt having Mw 13.000 Da, are introduced into the irradiation system formed of a reservoir, a circuit of 4 lamps (total W460), a peristaltic pump circulating the liquid into the circuit (19 l/h), and a small heat exchanger refrigerating the solution to 30 °C.

The liquid is irradiated for 16 hours.

15 Heparin is collected, spray-dried and analysed.

Mw = 5.000

Absorbance at 260 nm (solution 0.4%) = 0.080

Inorganic sulphates = absent

aXa activity = 106 U/mg

20 Anticoagulant activity = 114 U/mg

NMR: values obtained by intregation of <sup>13</sup>C-NMR signals

Sample	A <sub>NS6X-G</sub>	A <sub>NS6X-I2X</sub>	A <sub>NAc</sub>	A <sub>N,3,6S</sub>	A <sub>6S</sub>	I <sub>2S</sub>	I <sub>2OH</sub>	G
Starting heparin	12.92	68.08	13.6	4.6	75.9	61.4	13.26	25.7
Depolym. heparin	13.6	64.18	15.8	6.4	77.9	60.8	17.2	22

The obtained intermediate depolymerised heparin can be fractionated by Gel Permeation Chromatography. The fractions containing the desired molecular weights  
25 are collected, concentrated by ultra filtration and lyophilized.



**Example 2**

Example 1 was repeated but continuously filtering the liquid with a membrane having a cut off value of 5.000 Da. The liquid removed by membrane filtration is continuously integrated by addition of starting 10% heparin-Na salt solution.

5

**Example 3**

Example 1 was repeated but using a 5 % dermatan sulphate ( $M_w = 35.000$  Da) solution instead of a 10% heparin solution.

After irradiation, the recovered dermatan sulphate had the following characteristics:

10  $M_w = 15.000$  Da

Abs 260 nm = 0.150

Inorganic sulphates = absent

The obtained intermediate depolymerised dermatan sulphate can be fractionated by Gel Permeation Chromatography. The fractions containing the desired molecular weights

15 are collected, concentrated by ultra filtration and lyophilized.

## Claims

1. Process for the depolymerization of glycosaminoglycans to obtain depolymerised glycosaminoglycans having a  $M_w$  equal to or lower than 50% of the original  $M_w$  prior to depolymerization, characterized by the use of UV radiation having a peak in the range from 245 nm to 260 nm
2. Process according to claim 1 wherein the source of UV radiation is a medium or low pressure Hg lamp.
3. Process according to claims 1-2 wherein the UV radiation has a prevalent emission band at 253 nm.
4. Process according to claims 1-3 wherein the glycosaminoglycane is heparin.
5. Process according to claims 1-3 wherein the glycosaminoglycane is dermatansulphate.
6. Process according to claims 1-5 wherein the solution subjected to depolymerization is kept at a temperature comprised between 10 and 50 °C.
7. Depolymerized glycosaminoglycane obtainable according to the process of claims 1-6

**Abstract****Process for the physical depolymerization of glycosaminoglycans and products obtained therefrom.**

5

The invention relates to a process for the depolymerization of glycosaminoglycans characterized by the use of UVC radiation.

The invention also relates to the intermediate depolymerized heparin obtained by the process.

- 10 The intermediate depolymerized heparin can be dissolved in a buffer solution and fractionated by Gel Permeation for obtaining the desired Molecular Weight.

*0001* CT/EP2004/050723



**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**